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Artesunate Ameliorates Experimental Autoimmune Encephalomyelitis by Inhibiting Leukocyte Migration to the Central Nervous System

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SUMMARY

Background and Aims: Experimental autoimmune encephalomyelitis (EAE) is T-cell-dependent disease of the central nervous system (CNS) of mice. This model resembles multiple sclerosis (MS) in many aspects. Therapies that focus in the modulation of the immune response and cellular infiltration in the CNS present best effects in the clinics. Artesunate (Art) is a semi-synthetic sesquiterpene derivative from artemisinin and has been shown to reduce the clinical signs of autoimmune disease models through mechanisms not yet understood. In this study, we aimed to evaluate whether administration of Art would ameliorate EAE. **Methods and Results:** C57BL6 mice were immunized with MOG_{35-55} peptide to induce EAE. At the same time, Art treatment started (3 mg/kg/day via i.p.) for five consecutive days. We found that Art treatment reduced the clinical signs of EAE and that correlated with a reduced infiltration of cells in the CNS. Disease amelioration did not correlate with immunomodulation as recall responses, leukocyte subpopulations, and gene expression analysis were similar among treated and untreated mice. Ultimately, further analysis provided data indicating that a possible mechanism of action for Art is dependent on the cellular migration to the CNS. **Conclusions:** Artesunate reduces the severity of EAE by inhibiting migration of pathogenic T cells to the CNS.

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Introduction

Multiple sclerosis (MS) is a human demyelinating disease characterized by clinical signs that include paralysis, ataxia, and even death. MS is believed to affect more than 2 million people worldwide. The etiology of the disease is not completely understood but self-aggression against the components of the central nervous system (CNS) by leukocytes is believed to play a major role [1,2]. There are three main types of manifestation of MS: primary progressive (PP-MS), secondary progressive (SP-MS), and relapsing–remitting (RR-MS) [3]. In all forms, the outbreak of symptoms is associated with migration of encephalitogenic leukocytes to the CNS [4]. Experimental autoimmune encephalomyelitis (EAE) is a T-cell-mediated disease that shares many aspects with MS [5–7]. Although interferon-gamma-producing helper 1 T (Th1) cells are important orchestrators of inflammation, EAE is also dependent on IL-17-producing Th17 cells, where ablation of IL-17 production/signaling ameliorates disease [8–13]. The inflammation caused by infiltrating T cells worsens the disease. In this context, the administration of migration inhibitors, such as fingolimod and natalizumab, has been shown to improve patients' conditions [14–17]. These observations reinforce the need for the development of new drugs that target cell migration to the CNS in MS [18].

Many drugs that were used in the treatment of infections have discovered new uses in the amelioration of inflammation, which is the case of chloroquine, primaquine, dihydroxyartemisinin, and artesunate, among others [19–24]. Artesunate (Art), a semi-synthetic sesquiterpene derived from *Artemisia annua* L., has originally been used in the treatment of malaria [25,26]. Studies have shown that Art treatment reduced the severity of autoimmune diseases, such as rheumatoid arthritis, lupus, and ulcerative colitis [27–29]. Although the precise mechanism of action exerted by Art remains to be elucidated, it has been observed that Art treatment reduced the secretion of mediators of inflammation as inflammatory cytokines (IL-17, TNF-α, IFN-γ, IL-6), germinal center B cells, and proliferation of T cells [27–29].

In this study, we aimed to evaluate whether artesunate administration would reduce the clinical signs of EAE. We found that

mice receiving Art in a therapeutic regimen presented a mild EAE compared with untreated mice. Although the leukocytes subsets were investigated, we found that Art treatment does not alter the frequency of key cellular subsets of the EAE-mediated inflammation. Interestingly, expression of chemokine receptors was found reduced in T cells as well as the migratory ability of leukocytes from Art-treated mice. Taken together, our data present a novel mechanism of action exerted by artesunate: the inhibition of the migratory ability of inflammatory cells to the CNS of EAE-inflicted mice.

Materials and Methods

Animals

In total, 6- to 8-week-old female C57BL/6 and RAG2$^{-/-}$ mice were acquired from Centro Multidisciplinar para Investigação Biológica na Área da Ciência de Animais de Laboratório (CEMIB, University of Campinas). Mice were allocated in *specific pathogen-free* conditions, in cages (maximum 5 mice per cage) with free food and autoclaved tap water in a controlled photoperiod (12-h/12-h dark/light cycle) environment. All procedures were performed in ways that minimize animal suffering. The institutional ethics committee (University of Campinas Committee on the Use and Care of Animals—CEUA) approved all experiments under registration #3345-1(A).

Experimental Autoimmune Encephalomyelitis induction

For active EAE

Disease was induced in mice according to a previously described protocol [30]. Briefly, mice were immunized with 100 μg of MOG_{35-55} peptide (Genscript, Piscataway Township, NJ, USA) emulsified in complete Freund's adjuvant (CFA; Sigma-Aldrich, St Louis, MO, USA) through s.c. injection in the tail base. About 200 ng of pertussis toxin (Ptx; Sigma) was administrated via i.p. at 0 and 48 h following immunization. Mice were accompanied daily for the development of EAE. The clinical signs were evaluated based on score, in which: 0, no signs of inflammation; 1, limp tail; 2, limp tail and weakness of the hind limb; 3, paralysis of hind limb; 4; paralysis of hind limb and weakness of fore limb; and 5, full paralysis/dead.

For passive EAE

C57BL/6 mice were immunized with 100 μg of MOG_{35-55} peptide emulsified in CFA through s.c. injection. After 8 days of immunization, mice were killed and erythrocyte-free splenocytes were prepared. 5×10^6 cells were transferred to each RAG2$^{-/-}$ mice through the i.v. route. About 200 ng of Ptx was administrated via i.p. at 0 and 48 h following adoptive cell transfer. Mice were accompanied daily for the development of EAE. The clinical signs were evaluated as mentioned above.

Artesunate Treatment

Artesunate treatment followed directions from the World Health Organization [31], with modifications. Artesunate (Sigma) was dissolved in phosphate-buffered saline (PBS) 0.02M pH7.2. In all treatment regimens, Art was administrated via i.p. for five consecutive days. Each mouse was treated with Art at a concentration of 3 mg/kg/dose. For evaluation of Art on EAE severity, treatment started on the day EAE was induced. For therapeutic treatment, Art was administrated from day 10 to day 14 after immunization. For prophylactic treatment, Art was administrated starting 7 days before EAE induction. Artesunate half-life after ingestion has been determined elsewhere [32].

Analysis of the Cellular Infiltration in the CNS

The analysis of cellular infiltration in the CNS of EAE-bearing mice was performed according to a previously described methodology with small modifications [33]. Briefly, at the end of the experiment, mice were killed and the CNS was removed and disrupted in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). The suspension was filtered in 70-μm cell strainer (BD Biosciences, Franklin Lakes, NJ, USA) and enriched in isotonic Percoll (Sigma, GER) gradient (70/30) centrifugation (300 g for 30 min at 4°C without brakes). Cells from the interface were collected and washed in FACS staining buffer (PBS + 3% FCS). Cellular concentration was calculated with the aid of a hemocytometer. Samples were submitted to flow cytometry staining or otherwise stated.

Flow Cytometry Analysis of Cellular Subpopulations

Single-cell suspensions were prepared in FACS staining buffer (PBS + 3% FCS). 1×10^6 cells were transferred to conical tubes (12 × 75 mm; BD Biosciences) and incubated with fluorochrome-conjugated antibodies at a final volume of 100 μL for 20 min at 4°C (1 μg antibody/1 million cells). For intracellular staining of cytokines, 1×10^6 cells from each mice were incubated for 4 h with phorbol-12-myristate-13-acetate (PMA, 50 ng/mL; Sigma) and ionomycin (500 ng/mL; Sigma) in the presence of brefeldin A (BFA, 1 μg/mL; BD Biosciences). Later, cells were surface stained with monoclonal antibodies and permeabilized with Fix/Perm buffer (eBioscience, San Diego, CA, USA). The intracellular antigens were stained with fluorochrome-conjugated antibodies for 20 min at room temperature. Antibodies used in this study were anti-CD3e (clone 145-2C11), anti-NK1.1 (clone PK136), anti-CD11c (clone N418), anti-CD4 (clone GK1.5), anti-CD8 (clone 53-6.7), anti-CCR7 (clone 4B12), anti-CXCR4 (clone 2B11), anti-CCR9 (clone eBioCW-1.2), anti-CD25 (clone PC61), anti-Foxp3 (clone FJK-16s), anti-IL-10 (clone JES5-16E3), anti-IFN-γ (clone XMG1.2), and anti-IL-17 (clone eBio17B7). All antibodies were from eBiosciences. Preparations (at least 100.000 events) were acquired on Gallios flow cytometer (Becman Coulter, Inc, Jersey City, NJ, USA). Data were analyzed with FlowJo VX platform (Tristar Inc, Flow Jo LLC, Ashland, OR, USA).

Gene Expression Analysis

Gene expression analysis was performed as previously described [34]. Briefly, RNA was extracted from tissues using TRIzol reagent according to manufacturer's recommendations (Life Technologies, Carlsbad, CA, USA). About 1 μg of pure RNA was used to prepare

cDNA according to manufacturer's recommendations (High-Capacity kit; Life Technologies). RT-PCRs were conducted with Taqman reagents according to manufacturer's protocol (Life Technologies). Genes investigated in this study were CCL19 (Mm00839967_g1), CCR7 (Mm99999130_s1), IL-10 (Mm01288386_m1), Foxp3 (Mm00475162_m1), and IL-17 (Mm00439618_m1). Results are shown as relative expression ($2^{-\Delta\Delta C}$T).

Recall Response Assays

Analysis of specific cellular proliferation was conducted as previously described [35]. Briefly, mice were killed at determined time-points and draining lymph nodes were collected and disrupted to prepare single-cell suspensions. Cells (5×10^5) were seeded to each well of a 96-flat bottom well plate with RPMI 1640 medium supplemented with 10% FCS. For specific proliferation, cells were cultured in the presence of several concentrations of MOG_{35-55} peptide (Genscript). The plates were incubated for 96 h at 37°C. After the incubation period, the cultures were treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) for 4 h and the formazan crystals dissolved with isopropanol. Absorbances were read at 540 nm by spectrophotometer.

Migration Assays

Evaluation of the cellular migration ability was performed in 24-transwell plates containing inserts with a pore size of 5 μm (Corning Life Sciences, Pittston, PA, USA). Briefly, 5×10^5 cells from draining lymph nodes were seeded in the upper chamber in a final volume of 100 μL in RPMI 1640 supplemented with 10% FCS. The lower chamber was filled with 500 μL of medium containing recombinant mouse 10 ng of CCL19 chemokine (R&D Systems Inc., Minneapolis, MN, USA). As controls, some cells in the lower chamber received only medium. The plates were incubated for 3 h at 37°C. At the end of culture time, the cells in the lower chamber were counted in hemocytometer. In a set of experiments, cells from EAE-bearing mice were allowed to migrate in the presence of artesunate (2.5 μM; Sigma).

Statistical Analysis

For clinical scores of EAE, data were analyzed by two-way ANOVA with Bonferroni post-test. Analysis with three or more groups was performed with one-way ANOVA and Bonferroni post-test. Analysis between two groups was performed with Student's *t*-test. Results are expressed as mean ± standard error mean (SEM), unless otherwise stated. All analyses were carried out in GraphPad Prism software (version 6.0, GraphPad Software Inc., La Jolla, CA, USA).

Results

Amelioration of EAE in Art-Treated Mice Does not Correlate with Altered Leukocyte Subpopulations that Infiltrate the CNS

To evaluate whether artesunate presents a protective effect in EAE, we treated C57BL/6 female mice with Art (3 mg/kg) at the same time of MOG_{35-55} immunization. In this study, Art treatment is composed of five consecutive doses administrated on a daily basis. We found that Art treatment reduced the

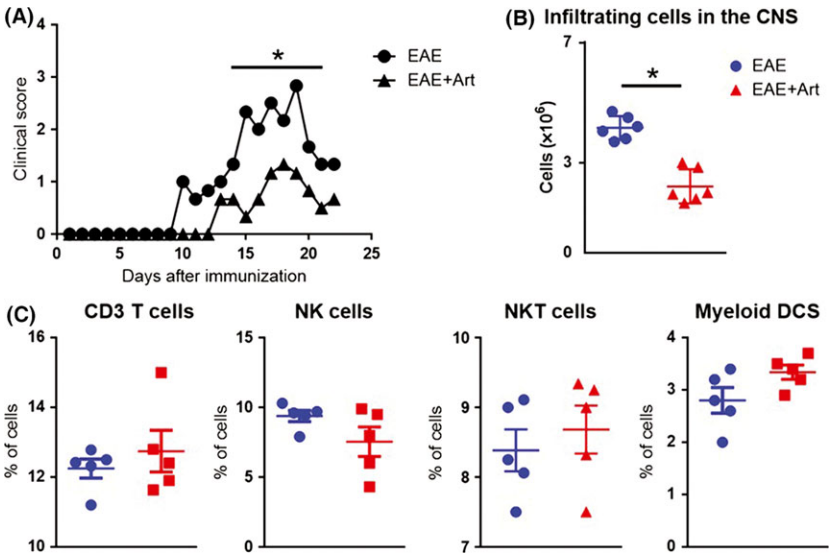

Figure 1 Treatment with artesunate reduces the severity of EAE. Experimental autoimmune encephalomyelitis (EAE) was induced in C57BL/6 mice (n = 10) through the immunization with 100 μg of MOG_{35-55} peptide and 200 ng/mice of pertussis toxin injection at 0 and 48 h from immunization. Mice were divided in two groups, where one group received artesunate (3 mg/kg) for five consecutive days starting the same day of immunization and the other group received vehicle (PBS 0.02M pH7.4). (**A**) The clinical score was assessed daily and shows that Art-treated mice developed milder EAE. Results are shown as group mean for each time-point. *$P < 0.05$ in two-way ANOVA multiple comparison test. (**B**) Cellular infiltration in the CNS of EAE-bearing mice was determined after Percoll gradient enrichment of leukocytes and shows reduced infiltration of cells in the CNS of Art-treated mice. *$P < 0.05$ in *t*-test analysis. (**C**) Flow cytometry analysis of the subpopulations of infiltrating cells of the CNS. Results from four independent experiments with similar observations.

overall severity of EAE compared with untreated mice (Figure 1A). Disease amelioration correlated with a reduction in the infiltration of leukocytes in the CNS of EAE-bearing mice (Figure 1B). Although the absolute number of infiltrating cells was reduced in EAE + Art mice, there was no alterations in the frequency of leukocyte subpopulations that infiltrated the CNS among groups (Figure 1C).

Artesunate Increased the Frequency of IL-10-Producing T Cells, Altered the Cellular and Tissue Expression of Chemokines, yet Lymphocytes Remained Responsive to Encephalitogenic Stimulus

As Art treatment did not change the frequency of leukocytes in the CNS, albeit reduced total infiltration, we aimed to evaluate whether the cytokine secretion profile of $CD4^+$ T cells was altered after treatment. We found that the frequency of IL-10-producing $CD4^+$ T cells doubled in Art-treated compared with untreated EAE-bearing mice (Figure 2A). Frequencies of IFN-γ- and IL-17-producing $CD4^+$ T cells and regulatory T cells were unaltered after Art treatment (Figure 2A). Of note, the protein expression of the chemokine receptors CCR7 and CXCR4, but not CCR9, in $CD3^+$ T cells was reduced in treated mice (Figure 2B). As it seemed that components of the cellular migration presented alterations, we aimed to evaluate the gene expression of chemokine receptors and ligands as well as mediators of inflammation in the CNS at the peak of disease severity (14 dpi) and in draining lymph nodes (dLN) in the preclinical stage of disease (10 dpi). We found that CNS from Art-treated mice presented a reduction in the expression of CCR7 and IL-17 compared to untreated mice while gene expression levels of CCL19, IL-10, and Foxp3 remained unaltered (Figure 2C). Interestingly, there was no significant change in the expression of these parameters in dLN from treated and untreated mice, with the exception of Foxp3 (Figure 2C). The results suggest that the cellular migration toward the CNS may be compromised after artesunate treatment. Indeed, we found an augmented cellularity of spleens and dLN from Art + EAE mice compared with untreated EAE mice after 20 days from $MOG_{35–55}$ immunization (Figure 2D). To test whether Art treatment modified the cellular

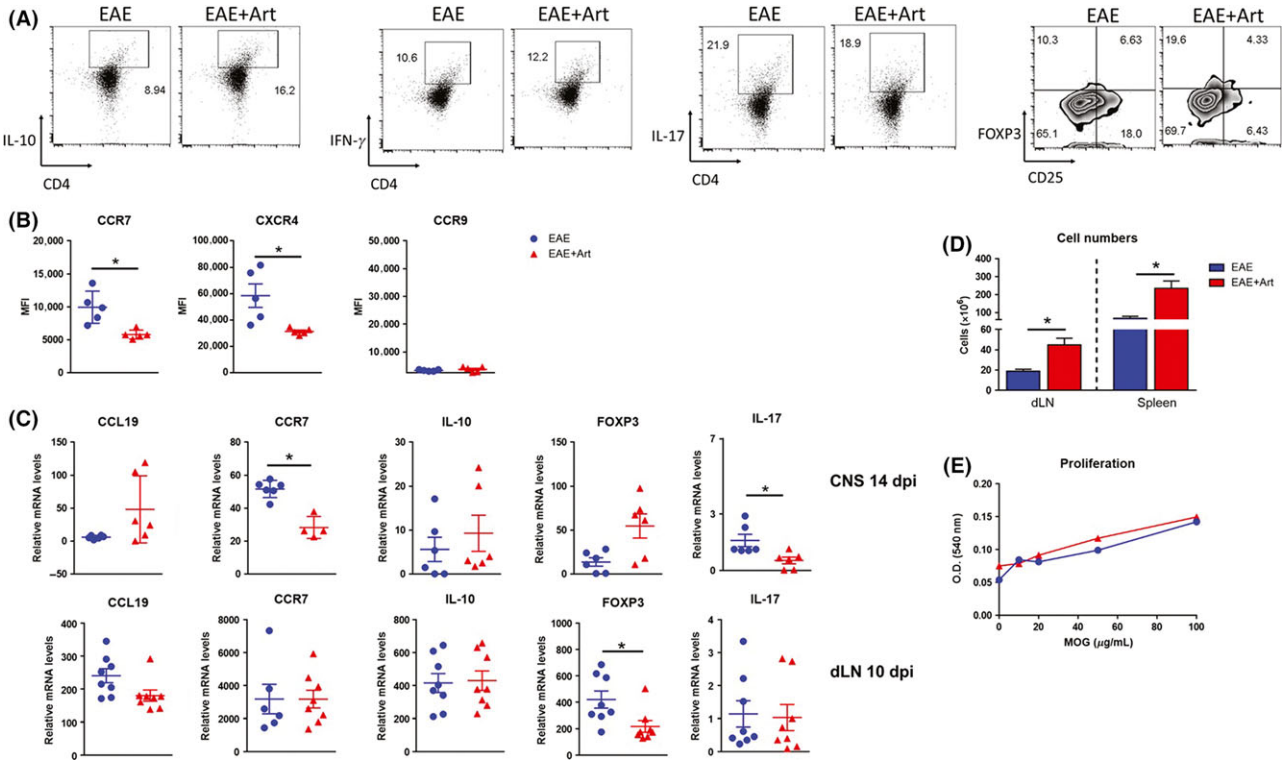

Figure 2 EAE amelioration in Art-treated mice correlates with leukocyte retention in secondary lymphoid organs but not with altered lymphocyte subpopulations in the CNS. EAE-bearing mice (n = 5/each group) were killed 20 days after immunization, and the brain and lumbar spinal cords were collected and submitted to gradient centrifugation to enrich infiltrating leukocytes. (**A**) To detect intracellular cytokines, cells were activated with PMA and ionomycin in the presence of brefeldin A (50 ng/mL, 500 ng/mL, and 1 μg/mL, respectively) for 4 h at 37°C. Later, cells were surface stained and permeabilized for intracellular staining. (**B**) Infiltrating T cells were surface stained for the analysis of CCR7, CXCR4, and CCR9 by flow cytometry. (**C**) CNS was collected from EAE-bearing mice at 14 dpi, whereas draining lymph nodes were collected at 10 dpi. Tissues were submitted to RNA extraction and cDNA production. Analysis of genetic expression of genes of interest was carried out. *$P < 0.05$ in *t*-test analysis. (**D**) Twenty days after disease induction, mice were killed and the cellularity of spleens and draining lymph nodes were determined. *$P < 0.05$ in *t*-test analysis. (**E**) Ten days after MOG immunization, mice were killed and the cells from dLN were collected and cultivated in the presence of increasing doses of $MOG_{35–55}$. Cells were incubated for 96 h at 37°C. Proliferation was determined through MTT method. Combined results from five independent experiments with similar observations.

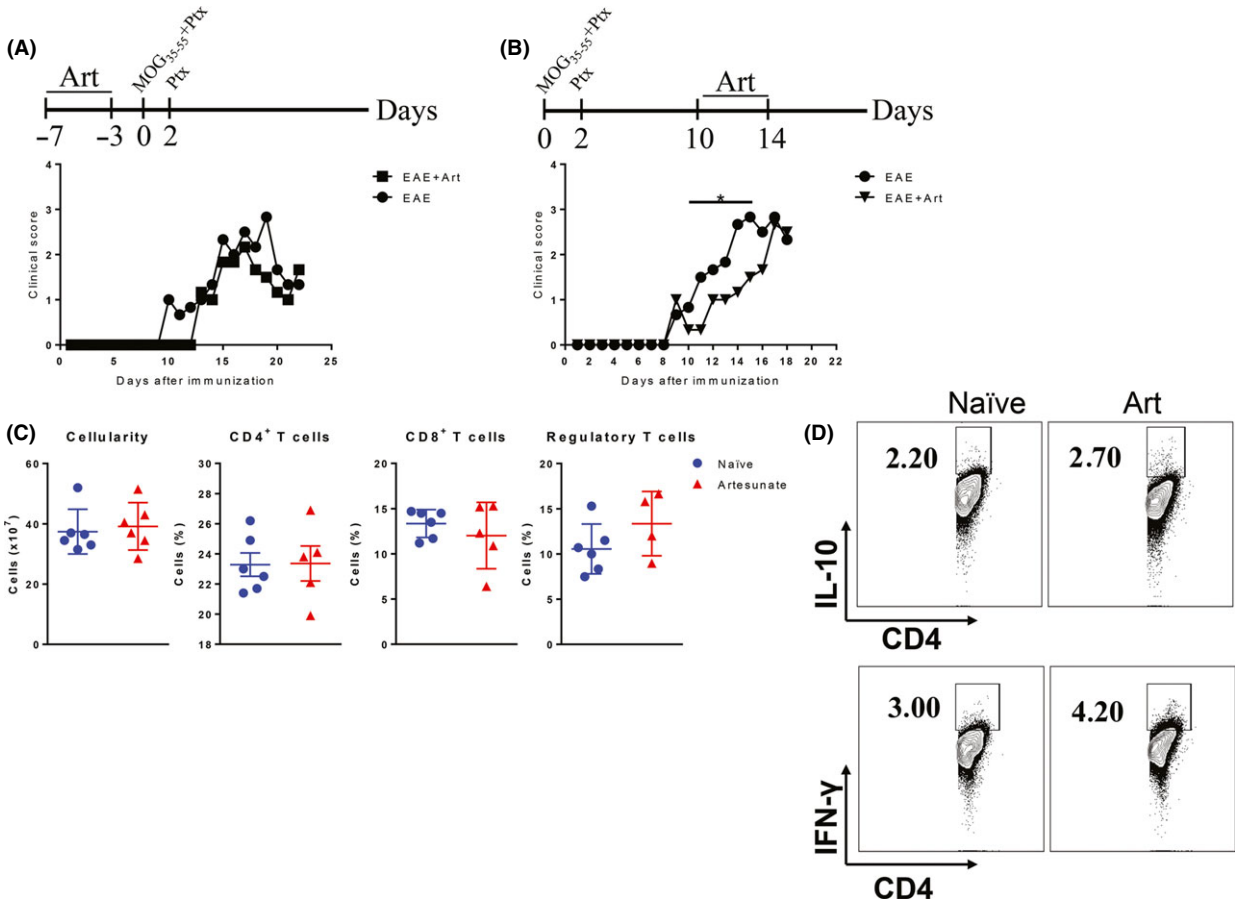

Figure 3 Administration of artesunate is only effective during inflammation. (**A**) Artesunate (3 mg/kg) was i.p. administrated to mice for five consecutive days in a prophylactic regimen starting 7 days before EAE induction. The clinical course of disease was evaluated daily and shows no alteration compared to untreated mice. (**B**) Art was administrated for five consecutive days starting on EAE onset (around day 10). Disease course was evaluated daily. *$P < 0.05$ in two-way ANOVA multiple comparison test. (**C**) Naïve mice were treated with Art via i.p. for five consecutive days. As controls, mice received PBS through the same route. At the last day of treatment, spleen cellularity and the T lymphocyte subpopulations were analyzed by flow cytometry. (**D**) Following Art treatment, naïve and treated mice were killed and the spleens were collected and cells were activated with PMA, ionomycing, and brefeldin A (50 ng/mL, 500 ng/mL, and 1 μg/mL, respectively) for 4 h. Later, surface and intracellular antigens were stained with fluorochrome-conjugated antibodies and analyzed by flow cytometry. Combined data from three independent experiments with similar results.

immune response to neuro-antigens, 10 days after immunization, we stimulated dLN-derived cells with increasing concentrations of $MOG_{35–55}$ and assessed the recall response. We found that lymphocytes from untreated and Art-treated mice retained their proliferative response toward the specific neuro-antigen (Figure 2D).

Artesunate is Efficient only When Administrated in Specific Time-Points During EAE Development

Our results so far showed that EAE-Art mice developed a mild EAE compared with untreated mice. We also found that the cellular immune response toward encephalitogenic peptide and the subpopulations the infiltrated the CNS remained unchanged in both groups. The main difference among treated and untreated mice was the reduced expression of chemokine receptors by T cells and the retention of cells in the secondary lymphoid organs in EAE + Art group. Therefore, we aimed to evaluate the "window of effectiveness" of Art. For that purpose, we started the treatment 1 week before EAE induction (Figure 3A) or at disease onset (Figure 3B). Our data showed that the prophylactic treatment was not efficient in reducing disease severity (Figure 3A). However, treatment at the start of the symptoms was effective and significantly reduced the severity of EAE (Figure 3B), although not to the same extent as when treatment started at day 0 of EAE (Figure 1A). To exclude the possibility of a potential immune-modulatory profile of Art, we treated naïve mice for five consecutive days and evaluated the subpopulations of T lymphocytes in secondary lymphoid organs as well as the production of cytokines by $CD4^+$ T cells. We found that treatment with Art did not change the cellularity of spleens nor did change the frequency of $CD4^+$, $CD8^+$ and regulatory T cells (Figure 3C). Intracellular production of IL-10 by naïve $CD4^+$ T cells was unaltered, which is in contrast to what we observed in infiltrating cells in the CNS of EAE + Art mice (Figure 2A). IFN-γ production presented the same pattern as of infiltrating cells in the CNS of EAE + Art (Figure 2A). These

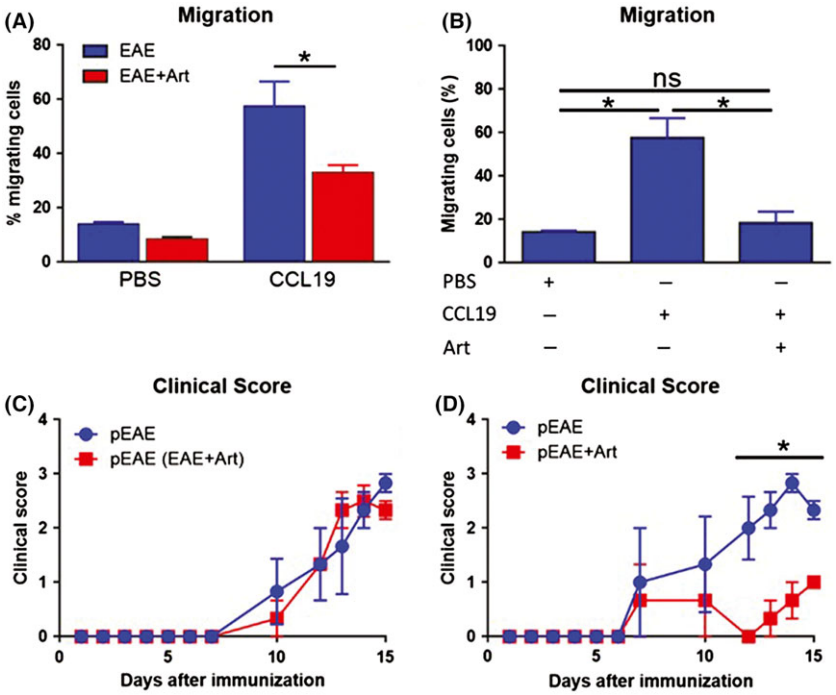

Figure 4 Artesunate reduces the migration of cells. Experimental autoimmune encephalomyelitis was induced in C57BL/6 mice (n = 10) through the immunization with 100 μg of MOG_{35-55} peptide and 200 ng/mice of pertussis toxin injection at 0 and 48 h from immunization. Mice were divided in two groups, where one group received artesunate (3 mg/kg) for five consecutive days starting the same day of immunization, and the other group received vehicle (PBS 0.02M pH7.4). (**A**) Ten days after immunization, mice were killed and the draining lymph nodes were collected. The organs were disrupted to prepare single-cell suspensions, which were seeded (5×10^5 cells) on the upper chamber of transwell plates. The lower chamber was filled with complete medium alone (PBS) or with recombinant mouse CCL19 (20 ng/mL). Cells were incubated for 3 h at 37°C. At the end of culture time, cells in the lower chamber were counted in hemocytometer. Results are presented as mean ± SEM. *$P < 0.05$ in *t*-test analysis. (**B**) Ten days after immunization, EAE-bearing mice were killed and the spleen cells (5×10^5 cells/insert) were seeded in the upper chamber of a transwell plate. In the lower chamber, RPMI medium alone (PBS) supplemented with CCL19 (20 ng/mL) with or without artesunate (2.5 μM). The cultures were incubated for 3 h at 37°C. At the end of incubation time, cells in the lower chamber were counted in hemocytometer. Results are presented as mean ± SEM. *$P < 0.05$ in one-way ANOVA multiple comparison analysis. (**C**) Spleen cells from untreated and Art-treated EAE-bearing mice at 10 dpi were adoptively transferred to $RAG2^{-/-}$ mice (5×10^6 cells/mice), and the clinical course of passive EAE was evaluated daily. Results are presented as mean ± SEM. (**D**) Spleen cells from untreated EAE-bearing mice at 10 dpi were adoptively transferred to RAG2−/− mice (n = 12 mice). Group of mice also received Art (3 mg/kg) for five consecutive days starting day 3 from adoptive transfer. The clinical course of passive EAE was evaluated daily. Results are presented as mean ± SEM. *$P < 0.05$ in two-way ANOVA multiple comparison analysis. Combined results from three independent experiments with similar observations.

results confirm that the beneficial effects of artesunate are obtained on a certain "window of opportunity" and that it is independent on an immune-modulatory activity.

Disease Amelioration in Artesunate-Treated Mice Relies on Inhibition of Leukocyte Migration

Our results suggest that the mechanism of action exerted by artesunate is dependent on the inhibition of leukocyte infiltration on the CNS rather than the modulation of the immune response. To test this hypothesis, we obtained dLN cells from EAE and EAE + Art mice and cultivated these cells for 3 h in transwell chambers in the presence of medium containing PBS or recombinant mouse CCL19. We observed that cells from EAE-Art mice presented a significant reduction in the migratory ability compared with EAE mice (Figure 4A). To evaluate whether Art would interfere with cellular migration by itself, without the involvement of an accessory cell or mechanism, we cultivated dLN cells from EAE mice for 3 h in transwell chambers with the same stimulus as before. However, some cells were cultivated in the presence of Art as well as CCL19. We found that the presence of Art in cultures significantly reduced the migration of encephalitogenic cells from EAE mice (Figure 4B). To confirm whether cells from EAE + Art mice retained their encephalitogenic potential, we induced passive EAE through the adoptive transfer of cells from EAE and EAE + Art mice. In concordance with our data so far, we found that mice developed passive EAE either if responder cells were from EAE or EAE + Art mice (Figure 4C). We did a final experiment to evaluate whether artesunate-induced EAE amelioration relied on an anti-migratory potential, and we adoptively transferred cells from EAE mice to $RAG2^{-/-}$ mice and treated these mice with Art 3 days later. In contrast to what we observed in mice recipient of EAE + Art cells, administration of Art to mice that received cells from untreated EAE mice led to a milder form of disease compared with untreated mice (Figure 4D).

Discussion

In this study, we show that artesunate present a beneficial effect on experimental autoimmune encephalomyelitis, model of MS, by interfering with the cellular infiltration in the CNS. Our data present a novel mechanism of action exerted by Art, the inhibition of cell migration rather than the modulation of the specific inflammatory response. These findings are interesting and shed light on a possible therapeutic potential of Art in the clinics of MS.

Multiple sclerosis is a human demyelinating disease that is dependent on the infiltration of inflammatory cells in the CNS where their products promote tissue damage and axonal death [1,36]. Therefore, therapies focused on cellular infiltration in the CNS are of great importance. We observed that Art treatment starting on the day of disease induction resulted in a mild form of EAE that did not correlate with altered cellular subpopulations or reduced cellular responsiveness to encephalitogenic peptide. Instead, we observed that EAE + Art mice presented higher numbers of cells in secondary lymph nodes compared to untreated EAE mice. This is interesting as the migratory ability of leukocytes was reduced in these mice as well. Together, these observations suggest that Art acts through the inhibition of cellular migration to the sites of inflammation.

Other studies have shown that Art presents a beneficial effect on the treatment of experimental arthritis models [23,27,37–40]. For instance, Art treatment initiating in the induction phase of collagen-induced arthritis (CIA) promoted a significant amelioration in the inflammatory process of the cartilages and that effect correlated with a diminished infiltration of leukocytes to the joints [23,37]. Art treatment in CIA promoted a reduction in the serum levels of TNF-α and the chemotactic factor MCP-1 [40]. Fibroblast-like synoviocytes from rheumatoid arthritis patients produced significantly less IL-8 after Art treatment than untreated cells [39]. It was shown that Art inhibits NF-κB, mitogen-activated protein kinase and AKT signaling pathways in macrophage cell line Raw264.7 and human fibroblast-like synoviocytes [38,41]. Notwithstanding, germinal center B cells were reduced while discrete alterations were observed in T-cell subpopulations after Art treatment in the K/BxN mouse model of rheumatoid arthritis [27]. Similar results were observed in experimental models of lupus and colitis [28,29].

We did not find significant alterations in the frequency of Th1/Th17 and regulatory T cells following Art treatment. We did observe that IL-10-producing T cells were increased in the CNS of EAE + Art mice, but not in spleens from healthy mice treated with Art. One possible hypothesis is that after priming, T cells are re-activated locally in the CNS by resident cells or antigen-presenting cells [42–45]. That second activation may be necessary to establish the IL-10-producing cell phenotype in the infiltrating T cell from EAE + Art mice. Still, we did not follow that line of investigation in this paper. Of note, bone marrow-derived dendritic cells (DCs) treated with artesunate and stimulated with lipopolysaccharide showed similar activation profile (MHC-II^{high}CD80^{high}CD86^{high}) as untreated DCs (*data not shown*). These data suggest that Art has little effect on the activation of T cells and dendritic cells. Literature present data regarding alterations in macrophages, synoviocytes, and B lymphocytes followed Art treatment [23,27,39,41]. We did not evaluate these cellular components in our study, as EAE is mainly a T cell-driven disease, although macrophages play an important role.

The main finding in our study is the observation that cells from EAE + Art mice presented lower expression of chemokine receptors, reduced migratory ability, and did not leave the secondary lymphoid organs in the course of EAE. Similar results are found in mice and transplanted/MS patients treated with FTY720 (fingolimod) and FK506 (tacrolimus) [46–51]. In our study, we found that T cells maintained their encephalitogenic potential after Art treatment, as transfer of cells from EAE + Art mice to RAG2$^{-/-}$mice resulted in normal disease development. This observation is important, as Art efficacy may be temporary in MS patients. In this context, accumulating evidence suggest that fingolimod-treated MS patients that discontinued treatment may present relapses or rebounds [48,52–54]. Forty-day evaluation of EAE + Art mice showed that disease worsens after 30 days from treatment discontinuation to the same levels as untreated mice (*data not shown*). Still, our data show promising results regarding the use of artesunate in the treatment of relapsing forms of MS. New studies must be conducted to ascertain the extent of the cellular migration inhibition or the migration receptors involved in Art-induced EAE amelioration.

Conclusion

Taken together, our data show that Art present a beneficial effect on experimental autoimmune encephalomyelitis, model of MS. To our knowledge, we are the first to present a novel mechanism of action exerted by Art, the inhibition of the cellular migration to the CNS. New studies must be conducted to evaluate the full range of migration receptors and cellular subsets involved in Art-induced EAE amelioration.

Acknowledgments

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Conflict of Interest

The authors declare that no competing interests exist.

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